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Review

Viral Bcl-2 homologs and their role in virus replication and associated diseases

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Abstract

Cellular Bcl-2 family proteins regulate a critical step in the mammalian programmed cell death pathway by modulating mitochondrial permeability and function. Bcl-2 family proteins are also encoded by several large DNA viruses, including all known gamma herpesviruses, adenoviruses, and several other unrelated viruses. Viral Bcl-2 proteins can prevent cell death but often escape cellular regulatory mechanisms that govern their cellular counterparts. By evading the "altruistic" suicide of infected cells, viruses can ensure replication and propagation in the infected host, but sometimes in surprising ways. Many human cancers and other disorders are associated with viruses that encode Bcl-2 homologs. Here we consider the available mechanistic data for viral compared to cellular Bcl-2 protein function along with relevance to the virus life cycle and human disease states.

Keywords: Bcl-2; Apoptosis; Gamma herpesvirus; Adenovirus; Epstein–Barr; Kaposi's sarcoma

1. Introduction

Programmed cell death that is triggered by virus-infected cells can prevent completion of the viral replication cycle and the production of progeny virus that would otherwise spread to adjacent cells in the host. This mechanism may serve to protect lower organisms that lack cellular immune systems and that must rely on alternate defense mechanisms to fight off infections. Mammals also utilize programmed cell death to control virus infections, but the apoptotic response to viruses and other pathogens is complex and frequently even contributes to pathogenesis. Infected cells initiate programmed cell death internally by sensing the effects of virus intrusion, such as virus-induced activation of the cell cycle, but death of infected cells can also be initiated externally by components of both the innate and adaptive immune responses such as death receptor signaling or in some cases by virus binding to its host cell receptor [1]. Death receptor activation in some cell types leads to direct activation of a family of cysteine-dependent/aspartate-directed proteases called caspases that are primary effectors of mammalian apoptosis. In many cell types, however, caspase activation is launched by first releasing mitochondrial factors that catalyze formation of the caspase-activating apoptosome complex or that antagonize inhibitors of apoptosis proteins (IAPs) [2]. Inevitably, viruses have adapted numerous ways of circumventing this host response, including expression of soluble death receptor decoys, regulation of endogenous host death receptors and ligands, expression of inhibitors of caspase activation and activity, regulation of host Bcl-2 proteins, and, notably, expression of viral homologs of Bcl-2 [3–5].

In the nearly 20 years following the discovery of Bcl-2, the number of known mammalian family members has mushroomed and Bcl-2 family proteins are now defined by inclusion of at least one of four distinct Bcl-2 homology (BH) domains and the variable presence of a putative membrane-spanning region at the C-terminus [6]. Thus, the Bcl-2 family is a collection of proteins that generally share low amino acid sequence similarity. Cellular Bcl-2 family proteins with two or more BH domains are classified as anti-death (Bcl-2, Bcl-X_L) or pro-death (Bax, Bak). A

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subgroup of pro-death Bcl-2 family members contain only a single 9–12-amino-acid motif resembling the BH3 domain of other family members. The three-dimensional structure is known for only one of the cellular BH3-only proteins, Bid. Although Bid lacks significant overall amino acid sequence similarity to other Bcl-2 proteins, its three-dimensional structure exhibits the conserved Bcl-2 fold. However, it is not currently known if all of the proteins now classified as BH3-only Bcl-2 family proteins will also share structural similarity. Nevertheless, the BH3 domain of Bid is important and in some cases sufficient to trigger death. Obvious homologs of Bcl-2 are conserved in species ranging from nematodes (*Caenorhabditis elegans*) to humans, and Bcl-2 overexpression can protect against cell death in yeast and plants, suggesting a universal, highly conserved ability to regulate cell survival. Thus, it is not surprising that viruses have adapted this mechanism to control host cell death and promote propagation in the host. The finding that all sequenced gamma herpesviruses encode at least one homolog of Bcl-2 implies that these proteins play an indispensable role in the virus life cycle. Recognizable Bcl-2 homologs are also encoded by at least one avipoxvirus (fowlpox), asfivirus (African swine fever) and alpha herpesvirus (Marek's disease-like).

The best studied viral Bcl-2 family member is E1B 19K of adenoviruses [4]. Using E1B 19K as a query, a blastp search against the nonredundant database at the National Center for Biotechnology or more sensitive searches using PSI-BLAST [7] and a library of hidden Markov models at Pfam [8] do not reveal homology between E1B 19K and Bcl-2 proteins. Pairwise alignments of E1B 19K with viral Bcl-2 proteins [such as Kaposi's sarcoma virus Bcl-2 (KSbcl-2)], as well as database searches with putative BH domains, reveal expect values that are not statistically significant. While it is thus unlikely that E1B 19K and Bcl-2 proteins share a common ancestor (a definition of homology), it is clear that they share similar anti-apoptotic functions [9]. On this basis E1B 19K is often referred to as a functional ''homolog'' of Bcl-2. In addition, determination of the three-dimensional structure may ultimately reveal a folded structure similar to Bcl-2. Human cytomegalovirus (beta herpesvirus) also has incorporated a Bcl-2-like function into its genome (vMIA), indicating that the advantage conferred to gamma herpesviruses by viral Bcl-2 is probably not unique [10]. Here we use the term homolog to refer only to the group of viral Bcl-2 family proteins with obvious amino acid sequence similarity.

Bcl-2, the founding member of the Bcl-2 family of death regulators, was originally identified as an oncogene at t(14;18) translocation chromosomal breakpoints that occur in follicular B cell lymphomas and result in Bcl-2 overexpression [11–13]. Bcl-2 (B cell lymphoma-2) represented a new type of oncogene that appeared to promote tumor formation primarily through the extension of cell survival rather than increased cell proliferation [14–17]. Nucleotide sequence analysis revealed sequence similarity to a previously identified gene encoded by Epstein–Barr virus (EBV) called BHRF1 [18] and BHRF1 was subsequently shown to inhibit cell death [19]. Whether viral Bcl-2 homologs contribute to oncogenesis in humans in an analogous fashion to cellular Bcl-2 is not yet known but remains a possibility in several virus-associated tumors.

2. How do Bcl-2 proteins modulate the death pathway?

An understanding of how viral Bcl-2 proteins function to inhibit apoptosis and promote the virus life cycle necessitates examination of how host Bcl-2 family proteins regulate cell death. Although considerable progress has been made in recent years, the precise biochemical mechanisms by which any Bcl-2 family protein inhibits cell death are still unknown and remain the subject of lively debate. The first clue came with the identification of Bcl-2-associated X protein, Bax, that was originally suggested to heterodimerize with Bcl-2 [20]. This led to the proposal of the ''rheostat'' model of cell survival where the ratio of anti-apoptotic to pro-apoptotic Bcl-2 family members of a cell receiving a death stimulus ultimately determines cell fate [21]. The significance of direct binding interactions between Bax and Bcl-2 or Bcl-X_L in vivo has been called into question because of detergent effects on heterodimer formation [22], non-overlapping intracellular localizations of Bax and Bcl-2 [22], and the ability of Bcl-X_L mutants deficient in Bax-binding to inhibit apoptosis [23]. Nevertheless, the pro-apoptotic functions of Bax and its relative Bak are suppressed by the anti-apoptotic Bcl-2 proteins when co-expressed in cells. In fact, Bax and Bak have been suggested to be the primary targets (direct or indirect) of viral Bcl-2 homologs, but this has not been rigorously demonstrated in physiological settings. However, extensive analysis of adenovirus E1B 19K indicates functional interactions with Bax and Bak as detailed below [4].

The second major clue to the biochemical function of Bcl-2 family members followed from the discovery that the release of mitochondrial cytochrome *c* was important for apoptotic cell death [24]. Bcl-2 was found to inhibit cytochrome *c* release [25,26] while Bax directly promoted cytochrome *c* release from isolated mitochondria [27,28]. Understanding how Bax initiates cytochrome *c* release and how Bcl-2 inhibits release therefore also becomes important for assessing the anti-death function of viral Bcl-2 relatives. Activation of the pro-death activity of Bax and Bak can be achieved by the BH3-only molecule Bid once Bid has been activated by proteolysis [29–33], as well as by other means that are still under investigation. Oligomerization of at least four Bax molecules in the mitochondrial outer membrane is likely a process critical for the ability of Bax (and presumably Bak) to induce membrane permeabilization; however, the importance of very large (=260 kDa) oligomeric complexes of Bax that have been observed in apoptotic cells for the permeabilizing activity is still in question [34–36].

Although the possibility remains that the anti-death function of Bcl-2 and Bcl-X_L is related to their ability to heterodimerize with Bax and Bak, thereby preventing Bax/Bak homo-oligomerization and the release of cytochrome *c*, mutants of Bcl-X_L incapable of binding Bax or Bak are still capable of inhibiting apoptosis [23]. Those Bcl-X_L mutants retain the ability to bind truncated Bid (tBid) [37] and another BH3-only protein Bad [38], prompting a revision of the heterodimerization model to suggest that the sequestration of BH3-only death ligands rather than of Bax and Bak death effectors was the primary mechanism of protection by anti-apoptotic Bcl-2 proteins (Fig. 1A) [37]. A genetic knockout approach demonstrated that Bax and Bak are indeed downstream of BH3-only molecules in the programmed cell death cascade and essential for apoptosis in response to multiple death stimuli [37,39,40]. A variation of this heterodimerization model supported by Strasser and colleagues suggests that BH3-only protein binding to Bcl-2 and Bcl-X_L negates an inherent anti-apoptotic function of these molecules, rather than masking a pro-apoptotic function of the BH3-only molecule (Fig. 1B). One proposal for this constitutive anti-death activity of Bcl-2 and Bcl-X_L was to hold Bax and Bak in check, however, the mechanism by which this could potentially occur was clouded by the failure to observe stable Bax/Bak-Bcl-2/Bcl-X_L interactions in healthy cells.

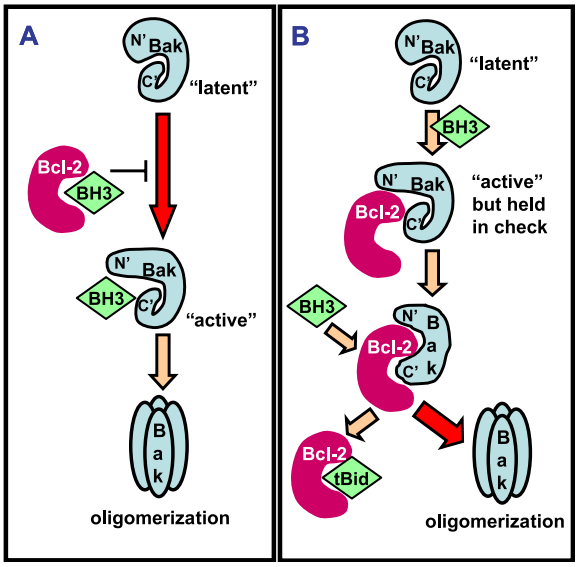


Fig. 1. Two models for the inhibition of Bax and Bak pro-apoptotic activity by anti-apoptotic Bcl-2 proteins. In both models, an active BH3-only molecule such as tBid initiates a sequential conformational change in Bak (or Bax) exposing the N-terminus followed by the C-terminus. For clarity, Bcl-2 and Bak are the examples used in the figure, but in these hypothetical models, Bak can be replaced by Bax, and Bcl-2 is representative of all anti-apoptotic proteins thought to function like Bcl-2. In (A), Bcl-2 does not interact with Bak but prevents BH3-only proteins from initiating the conformational change in Bak that leads to oligomerization and mitochondrial outer membrane permeabilization. In (B), Bcl-2 does not interact with Bak in the absence of an apoptotic stimulus, but following BH3 protein-induced conformational changes in Bak, Bcl-2 can bind the "active" conformations of Bak, preventing it from oligomerizing and increasing mitochondrial outer membrane permeability. In keeping with this model, the adenovirus Bcl-2 functional homolog E1B 19K does not interact with Bax unless an N-terminal conformational change first occurs. However, unlike cellular Bcl-2, E1B 19K cannot interact with tBid, although E1B 19K has been found to interact with other BH3-only proteins that may serve a similar role. The red arrow in each model indicates the step in the pathway that is blocked by Bcl-2.

Recent evidence in which chemical cross-linkers reveal that cellular Bcl-2 has a preferred affinity for an N-terminally-modified "active" conformation of Bak over a Bid BH3 peptide has prompted the revisiting of this idea that the primary function of Bcl-X_L and Bcl-2 is to hold Bax and Bak in check (Fig. 1B) [41]. This finding is reminiscent of the observation that adenovirus E1B 19K binds Bax only after Bax has undergone an N-terminal shift in conformation [42–45]. According to this revised model, an upstream signal such as tBid induces a conformational change in Bak (and presumably Bax) at the N-terminus that may precipitate a similar change in neighboring Bak molecules, leading to auto-oligomerization [41]. In this model, anti-apoptotic Bcl-2 inhibits oligomerization of Bak by binding the active Bak conformer, but this inhibition can be relieved if the concentration of BH3 death ligands is sufficiently high to sequester Bcl-2 away from Bak. The requirement of a conformational change for the interaction between Bak and Bcl-2 could explain why previously such interactions between Bax or Bak and antiapoptotic Bcl-2 family members were seen only in the presence of nonionic detergents [22,46].

It is important to note that the two models (Bax/Bak binding to Bcl-2 vs. Bax/Bak binding to BH3-only molecules that are "sequestered" by Bcl-2) are not mutually exclusive. Thus, cellular and viral Bcl-2 and perhaps other viral anti-apoptotic proteins could act both by inhibiting Bax/Bak and by sequestering BH3-only death molecules. Additionally, BH3-only proteins likely have roles in cell death that are independent of their ability to promote Bax/Bak activation that may also require opposition, such as the ability of tBid to alter inner mitochondrial membrane structure [47]. Because the majority of yeast two-hybrid, co-immunoprecipitation, and GST pull-down data on viral Bcl-2 protein interactions with host family members has focused on Bax and Bak over BH3-only molecules, it is difficult to conclude that viral Bcl-2 proteins primarily target the core apoptotic machinery rather than their upstream BH3 activators. For example, E1B 19K binds both BH3-only proteins (Bik [48,49] and Nip proteins such as BNIP3 [50]) and Bak [51] and activated Bax [52,53], while some viral Bcl-2 proteins often fail to bind Bax or Bak even in the presence of nonionic detergents. Thus, data for adenovirus E1B 19K favor the model of primarily targeting the core mitochondrial permeability regulators Bax and Bak for its mechanism of protection, but the role of heterodimerization with pro-apoptotic cellular Bcl-2 family members in protection by the herpesvirus and other viral Bcl-2 homologs remains largely untested.

A third critical clue regarding the biochemical functions of Bcl-2 family proteins was provided by three-dimensional structure determination. Bcl-X_L was found to have structural similarity to the B domains of bacterial toxins such as diphtheria toxin and colicins, which can form pores in lipid membranes and translocate toxin A domains across the membrane [54]. Both anti- and pro-apoptotic Bcl-2 family members form channels in artificial membranes [34,55–62] and modulate channels formed by the reconstituted mitochondrial proteins, adenine nucleotide translocator (ANT) [63] and voltage-dependent anion channel (VDAC), when added to membranes [64,65]. In the case of pro-apoptotic Bax and Bak, mitochondrial outer membrane channel formation has been proposed to mediate the release of intermembrane space proteins that have roles in apoptosis (e.g. cytochrome *c*, Smac/DIABLO, and AIF), although whether a protein or lipidic pore is responsible is still in question [2,66]. An alternative hypothesis suggests that Bax may contribute to activation of a mitochondrial inner membrane channel called the permeability transition pore that releases intermembrane proteins through matrix swelling and concomitant outer membrane rupture [63,67,68]. Thus, while the evidence for Bax/Bak-mediated release of mitochondrial proteins to promote death in apoptosis is now overwhelming, the biochemical mechanism by which Bax and Bak accomplish this feat is undefined and may differ among cell types and death stimuli (recently reviewed in Ref. [2]). In addition, a fraction of Bax and Bak proteins that localize to the endoplasmic reticulum (ER) was found to alter the steady-state level of ER calcium that, when released following a death stimulus, contributed to cell death [69,70]. Even more complicating is the observation that Bax and/or Bak functions as potent inhibitors of apoptotic neuronal death in mouse models of seizure and virus-induced encephalitis [71,72]. The ability of Bak to reduce neuronal excitability together with the observation that Bcl-X_L can enhance neuronal excitability suggest that the ability of Bcl-2 family proteins to regulate synaptic responses may contribute to their reversed phenotype in some neuron subtypes [72,73].

The function of channels formed by anti-apoptotic Bcl-2 proteins in the context of cell death is more opaque than for the pro-death family members Bax and Bak. The α5 and α6 helices of Bcl-2 were required for pore formation in artificial membranes and for protection against Bax-induced death, but not for Bax binding (via the tail of hydrophobic tail of Bcl-2), suggesting that channel formation may play a role in the anti-apoptotic mechanism of Bcl-2 [61,74]. A reduction in mitochondrial outer membrane permeability to ATP occurs in growth factor withdrawal-induced apoptosis that is rescued by Bcl-X_L or Bcl-2 overexpression [75]. That is, Bcl-X_L is suggested to maintain the flow of ATP into the cytosol following a death stimulus. Whether this reduction in permeability is a general feature of apoptosis and how it contributes to cell death progression remain to be determined. A further link between Bcl-2 family proteins and metabolism comes from the finding that the BH3-only protein Bad is required for assembly of a complex containing glucokinase at mitochondria [76]. Mice deficient for Bad lack this complex and exhibit characteristics of diabetes [76]. Thus, Bcl-2 family proteins likely have multiple biochemical functions/interactions, and these independent functions are difficult to study in isolation.

3. Structure and dimerization of Bcl-2 proteins

Analysis of the structure of Bcl-X_L complexed to a Bak BH3 peptide revealed that the BH1, BH2, and BH3 domains of Bcl-X_L surround a hydrophobic cleft that enables the binding of an α-helical BH3 domain of a binding partner [77]. Mutations in the BH1 and BH2 domains of Bcl-2 can abrogate its ability to interact with Bax and protect against apoptosis, indicating the importance of these domains for anti-death activity [78]. Mutation of the homologous BH1 residue in the African swine fever virus homolog LMW5-HL/A179L and BH1 and BH2 mutations in EBV BHRF1 also nullified anti-death activity, suggesting functional conservation between viral and cellular BH1 and BH2 domains [79,80]. Not all BH1 mutants lose the ability to protect, however, a BH1 mutant of Bcl-X_L retains the ability to bind tBid and inhibit apoptosis despite the loss of Bax binding [23,37]. The importance of the BH1 and BH2 domains of viral Bcl-2 homologs for their binding interactions with Bax/Bak vs. BH3-only molecules has yet to be fully tested. All viral Bcl-2 structural homologs contain a recognizable BH1 domain and with the exception of γHV68 Bcl-2, a BH2 domain [5]. However, the BH3 and BH4 motifs are poorly conserved, possibly indicating that these domains are less important for anti-apoptotic activity, or that these modified viral domains possess novel functions. Three-dimensional structure determination of E1B 19K is expected to confirm that this protein has domains that are positionally analogous to BH1 and BH2.

The three-dimensional structure of KSBcl-2 revealed overall structural similarity to the Bcl-2 family proteins Bcl-X_L, Bcl-2, Bcl-w, Bax, and Bid, even in domains where the primary amino acid sequence is not conserved [54,81–86]. The reason that certain Bcl-2 family members (e.g. Bax) behave largely as pro-apoptotic proteins while others (e.g. Bcl-X_L) are predominantly anti-apoptotic despite gross structural similarity is unclear but may be related to their propensity to form large oligomeric complexes [87]. The α-helices 5 and 6 that are required for Bcl-2 pore formation [61,74] are preserved in the KSBcl-2 structure, suggesting that like Bcl-2, KSBcl-2 might also form pores in membranes, although this has yet to be tested. As was the case for the three-dimensional structure of Bcl-X_L bound to a BH3 peptide of Bak, the BH1-BH2 domains together with the alpha helix corresponding to BH3 in the KSBcl-2 protein are involved in binding a BH3 peptide derived from Bak [81]. In contrast to Bcl-X_L, however, which displays

the greatest binding specificity for the BH3 domain of Bad (K_d = 0.6 nM vs. K_d = 3,900 nM for KSBcl-2), KSBcl-2 was much more proficient in binding Bak BH3 (K_d < 50 nM vs. K_d = 480 nM for Bcl-X_L) and to a lesser extent Bax BH3 (K_d = 980 nM vs. K_d = 13,000 nM for Bcl-X_L) [81,82,88].

This predicted binding specificity of KSBcl-2 for Bcl-2 proteins supports the concept that viral Bcl-2 proteins target the pivotal death effectors Bax and Bak rather than tissue and death stimuli-specific BH3 molecules for their inhibitory action. However, no interaction between KSBcl-2 and Bax or Bak was detected using in vitro translated proteins [89]. These findings could be reconciled if a conformational change in Bak or Bax is required for interaction with KSBcl-2 as was the case for the interaction between Bax and adenovirus E1B 19K [42–45]. Another reasonable possibility is that that the C-terminal alpha helix of KSBcl-2 is folded into the hydrophobic BH3-binding cleft as was found for Bax [84] and Bcl-w [85,86]. This C-terminal tail would need to be released to reveal the hydrophobic binding pocket and enable interactions with partner proteins. Unlike KSBcl-2, the herpesvirus saimiri Bcl-2 homolog ORF16 interacts with both Bax and Bak in GST pull-down assays [90].

In contrast to KSBcl-2 and cellular Bcl-2, the solution structure of EBV BHRF1 revealed that although the gern-eral alpha-helical folds typical of Bcl-2 family members were conserved in this viral protein, the prominent binding groove found in the cellular Bcl-2 homologs was absent [91]. Notably, three nonpolar residues found in the groove of Bcl-X_L and KSBcl-2 that are believed to contact a bound Bak BH3 peptide are replaced by polar residues in BHRF1. In this respect, BHRF1 may be more like Bid, which also lacks the deep cleft present in other family members [92]. Consistent with the absence of a hydrophobic BH3-binding pocket, BHRF1 did not bind Bak, Bax, or Bad BH3 peptides and bound to a BH3 peptide of Bid only weakly. However, EBV BHRF1 does bind Bak, but not Bax in GST pull-down, immunoprecipitation, and yeast two-hybrid studies [90,93], indicating that the ability to interact with synthetic BH3 domains may not always be predictive of the capacity to interact with whole proteins and raising the possibility that domains other than the BH3 domain of Bak are important for the observed interaction. Another possible heterodimerization domain is the BHRF1 hydrophobic tail (not included in the available structure), which could occupy the hydrophobic cleft of another family member. Interestingly, we have found that purified recombinant BHRF1, in contrast to recombinant human Bcl-X_L, does not inhibit tBid-induced cytochrome *c* release from isolated brain mitochondria even though BHRF1 associated avidly with mitochondrial membranes (Fig. 2), supporting the idea that viral Bcl-2 family members may employ non-heterodimerization-mediated mechanisms to block tBid and Bax/Bak function in cells.

Some gamma herpesviruses are difficult to propagate in cell culture, and the ability of their Bcl-2 homologs to bind

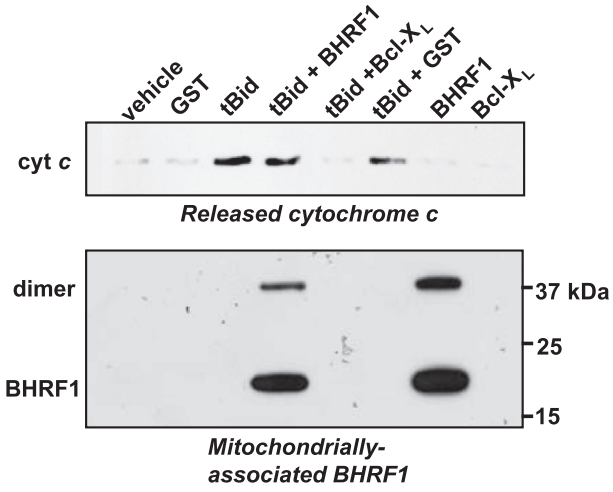


Fig. 2. Inhibition of tBid-induced cytochrome *c* release by recombinant human Bcl-X_L but not recombinant EBV BHRF1. Isolated brain mitochondria (0.5 mg/ml) were incubated in the presence of 125 mM KCl, 2 mM P_i, 20 mM HEPES, pH 7.0 with added 5 mM malate, 5 mM glutamate, 4 mM $MgCl_2$, 3 mM ATP, and 1 mM ADP at 30 °C. 50 nM tBid was used to stimulate cytochrome *c* release in the presence and absence of 500 nM Bcl-X_L, BHRF1, or GST control and released cytochrome *c* was immunodetected in the supernatant following centrifugation of the mitochondrial suspension. Mitochondrially associated EBV BHRF1 was detected by immunoblotting of pellets following centrifugation. No BHRF1 was detected in the supernatant.

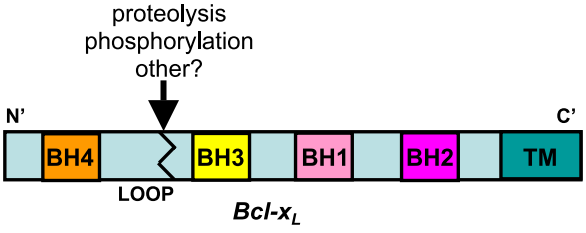
endogenous cellular Bcl-2 proteins during productive virus infections is difficult to assess. However, E1B 19K binds Bak in adenovirus-infected cells and prevents Bak–Bax interaction [44]. Despite the various data showing Bax and/or Bak interaction with viral Bcl-2 proteins, it is important to note that BHRF1 and KSBcl-2 were potently protective against Bax-induced cell death in transfected mammalian cells where stable interactions with Bax were not observed [94], suggesting that not all protection by viral Bcl-2 proteins can be explained via stable, direct sequestration models and additional mechanisms of anti-apoptotic activity remain to be discovered.

4. Posttranslational regulation of Bcl-2 family proteins

The composition and function of Bcl-2 proteins subcategorize them into three classes according to conventional dogma: (1) Multi-domain proapoptotic proteins (e.g. Bax and Bak), (2) multi-domain anti-apoptotic proteins (e.g. Bcl-X_L and Bcl-2), and (3) BH3 domain-only pro-apoptotic proteins (e.g. Bid and Bim). This conventional view has been challenged, however, by the findings that "anti-apoptotic" Bcl-2 and Bcl-X_L have latent pro-death activity that is activated by protease-mediated removal of the N-terminus [95,96] and that "pro-apoptotic" Bax and Bak display potent anti-death activity under some circumstances [71,72,97]. A revised model suggests that all Bcl-2 family proteins are inherently pro-survival (or neutral) with respect

to cell fate and require posttranslational events (e.g. proteolysis, phosphorylation, conformational changes or other) to uncover their pro-apoptotic function [5].

Cleavage of Bcl-2 and Bcl-X_L is not merely a fortuitous byproduct of caspase activation but is important for inactivating the survival function of these proteins to ensure cell death and in some cases may play an active role in the death process [95,96,98–100]. This presents a potential conundrum for the viral strategy of adapting Bcl-2 function for its own purposes, since direct caspase activation via death receptor stimulation by cytotoxic cytokines could nullify the protective function of viral Bcl-2 homologs. Phosphorylation is also known to impair the anti-apoptotic potency of Bcl-X_L and/or Bcl-2 [101–103], which might potentially target viral Bcl-2 as well. Viral Bcl-2 proteins have circumvented this problem, in part, by streamlining to include apparently the minimal domains required for protection while largely minimizing regulatory regions such as the loop domain between BH4 and BH3 that in their cellular counterparts is subject to phosphorylation and proteolysis (Fig. 3A). Out of six gamma herpesvirus Bcl-2 homologs tested, Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus 8 Bcl-2 (KSBcl-2), γ-herpesvirus 68 (γHV68) Bcl-2, herpesvirus saimiri (HVS) Bcl-2, EBV BHRF1 and BALF1, and bovine herpesvirus 4 (BHV4) Bcl-2, only γHV68 Bcl-2 could be cleaved by recombinant caspase-3 or apoptotic cell extracts (Fig. 3B), suggesting that with the exception of γHV68 Bcl-2, viral Bcl-2 proteins are resistant to this mechanism of inactivation [94,104].



Protein	Virus	Proteolysis	Latent Death Activity
KSBcl-2	Kaposi's sarcoma-associated herpesvirus	No	Yes
BHRF1	Epstein-Barr	No	No
BALF1	Epstein-Barr	No	No
γHV68 Bcl-2 (M11)	γ-herpesvirus 68	Yes	No
HVS Bcl-2 (ORF16)	herpesvirus saimiri	No	No
BHV4 Bcl-2	bovine herpesvirus 4	No	No

Fig. 3. Posttranslational modification of host vs. viral Bcl-2 family proteins. (A) Cellular Bcl-2 family members (i.e. Bcl-X_L) are subject to posttranslational modifications such as proteolysis and phosphorylation within the regulatory loop region that can inactivate anti-apoptotic and uncover latent pro-death activity. (B) Viral Bcl-2 homologs are resistant to inactivation by cleavage and have largely eliminated latent pro-apoptotic activity, perhaps by failing to conserve the BH3 "death" domain.

Resistance to proteolysis may be a contributing factor to the finding that BHRF1 can suppress cell death in some cases where Bcl-2 is ineffective [80]. Although γHV68 Bcl-2 is cleaved by caspase-3 and by apoptotic cell extracts, expression of an engineered "cleavage" product lacking its N-terminus could not induce apoptosis, indicating that the latent pro-death activity of Bcl-2 is not retained by the virus.

Even in the absence of proteolysis, it appears that the latent apoptosis-promoting activity of cellular Bcl-2 can sometimes be manifested, at least in overexpression assays [105]. It was therefore of interest to determine whether other viruses, like γHV68, have also eliminated this masked pro-apoptotic activity. Expression of other N-terminal truncated gamma herpesvirus Bcl-2 proteins, with the exception of ΔN20 KSBcl-2, failed to induce death of baby hamster kidney (BHK) cells [104]. This may be due to the observation that the BH3 "death" domain that is important for the latent pro-apoptotic activity of Bcl-2 is poorly conserved in many viral Bcl-2 homologs. Although a C-terminal KSBcl-2 fragment was capable of killing, this fragment is apparently not generated in healthy or apoptotic cells transfected with full-length KSBcl-2 even when in the same experiment the analogous expression of mammalian Bcl-2 results in cleavage and apoptosis [104]. Thus, by incorporating and subverting cellular Bcl-2 function to their own advantage, viruses have eliminated two key features of Bcl-2: inactivation of anti-death function by proteolysis and activation of latent pro-apoptotic activity. Interestingly, a cyclin homolog of Kaposi's sarcoma-associated herpesvirus directs cyclin-dependent kinase 6 (cdk6) to phosphorylate and inactivate host Bcl-2 while leaving the function of KSBcl-2 unaffected [102], indicating that there may be additional reasons why viruses have adopted their own Bcl-2 homologs rather than making exclusive use of cellular proteins [106].

5. Role of viral Bcl-2 in virus replication and associated disease

While understanding the biochemical interactions and viral functions of Bcl-2 proteins is of fundamental importance, the larger picture regarding their role in the virus life cycle and associated diseases is even more complex. Evidence discussed in detail below points to functions for various family members in facilitating replication, reactivation from latency, and virus-associated tumorigenesis (Fig. 4). Several human tumors are associated with KSHV that expresses KSBcl-2 and with EBV that expresses BHRF1 and BALF1, but a potential role for these viral Bcl-2 homologs in human disease remains highly speculative in the absence of good animal models to address this question. The best evidence for an in vivo role for a viral Bcl-2 family member in pathogenesis arose from the study of murine γHV68 mutants with a disrupted γHV68 Bcl-2 gene. Although adenoviruses are not known to cause human tumors, the more tractable cell culture models with which

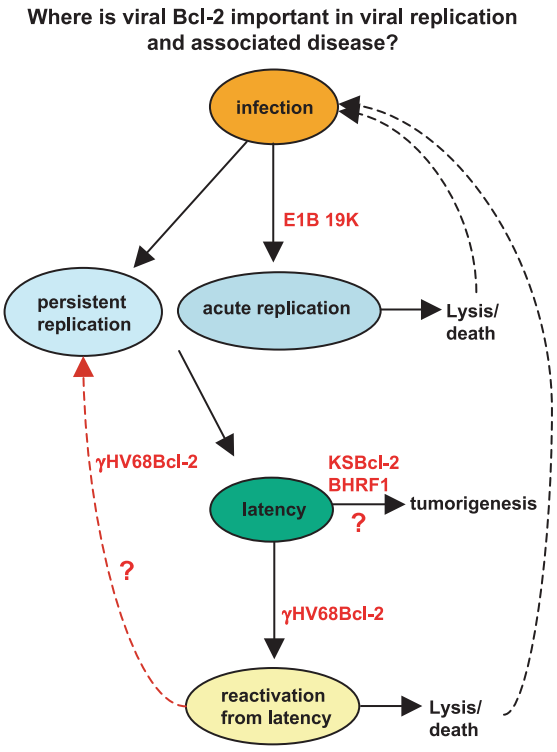


Fig. 4. Role of viral Bcl-2 in the virus life cycle. E1B 19K promotes acute replication by inhibiting apoptosis and preventing premature death of the infected cell. γHV68 Bcl-2 is suggested to promote reactivation from latency and persistent replication but has not been shown to play a role in acute replication or the establishment of latency. Further research is needed to elucidate the role of persistent replication in the gamma herpesvirus life cycle and associated disease states. KSBcl-2 and BHRF1 are speculated to participate in the tumorigenesis associated with their respective viruses by analogy to the function of Bcl-2 in follicular lymphomas and its conservation in clinical isolates, but because of the lack of good animal models this hypothesis has yet to be tested. Although E1B 19K can cooperate with adenovirus E1A in cell transformation, adenovirus infections are not associated with human cancers.

to study adenovirus deficient in E1B 19K have revealed a role for apoptosis in limiting viral replication and have elucidated host biochemical death pathways that respond to virus infection [44,107]. Adenoviruses have also served as a powerful model in which to study the role of anti-apoptotic E1B 19K in cellular transformation.

5.1. γHV68 Bcl-2

A Bcl-2 homolog is encoded by the M11 open reading frame of murine γHV68 [108]. γHV68 Bcl-2 (also referred to as M11) has a recognizable BH1 domain and hydrophobic C-terminal region, but lacks defined BH2, BH3 and BH4 domains [4,5,104,108,109]. Like cellular anti-apoptotic Bcl-2 proteins in some cell types, γHV68 Bcl-2 is protective against many death stimuli including Sindbis virus infection, Fas ligation and tumor necrosis factor α (TNF-α) treatment [104,109,110]. Surprisingly, despite the presence of a putative C-terminal transmembrane domain and in contrast to mammalian Bcl-2, hemagglutinin (HA)-tagged γHV68 Bcl-2 protein was predominantly cytoplasmic in transfected HeLa cells [109]. It is possible that the HA tag influences intracellular localization or that localization is influenced by the expression of other γHV68 proteins. However, it is also possible that γHV68 Bcl-2 has a non-mitochondrial site of action. The EBV Bcl-2 homolog BALF1 is also cytosolic [94], and mammalian Bcl-X_L is partially localized to the cytosol [111], supporting the notion that Bcl-2 family proteins have cytoplasmic targets in addition to their known activities at mitochondria and ER. Consistent with this idea, γHV68 Bcl-2 as well as BALF1, BHRF1, KSBcl-2 and Bcl-X_L bind the novel cell death regulator Aven that interacts with components of the apoptosome (Ref. [112] and DS Bellows, P Irusta, and JM Hardwick, unpublished observations).

γHV68 Bcl-2 mRNA had been detected both early in lytic/replicative infection cycle and in latently infected tissues [110,113], but until recently, any participation of γHV68 Bcl-2 in lytic replication and the establishment of latency was unknown. To address the function of viral Bcl-2 in the virus life cycle, recombinant γHV68 viruses lacking their Bcl-2 homolog were tested in an animal model to provide the first evidence that viral Bcl-2 plays a role in vivo [114]. Two independent mutant viruses were generated with premature stop codons in the γHV68 Bcl-2 gene, precluding translation of the majority of the sequence, including both the BH1 domain and hydrophobic C-terminal tail. Contrary to expectations, viral Bcl-2 was inconsequential to lytic replication as no differences in virus replication were observed in the spleen or liver during acute infection of mice with wild-type γHV68 vs. the mutant Bcl-2 viruses. Similar results were obtained in immunocompromised interferon-γ-deficient (IFN-γ $^{-}/_{-}$) mice [114]. These results are surprising in that inhibition of apoptosis is thought to promote acute replication by delaying the death of infected host cells. However gamma herpesviruses are known to encode multiple inhibitors of apoptosis, so it may be that γHV68 Bcl-2 is functionally redundant.

Subsequent to acute infection, gamma herpesviruses enter a latent state in which the viral genome exists as a circular episome. During latency a distinct subset of genes is transcribed. Since γHV68 Bcl-2 mRNA was detected in latently infected tissues, it is possible that γHV68 Bcl-2 might play a role either in the establishment of or re-emergence from latency. A functional γHV68 Bcl-2 was not required for the virus to establish latency in peritoneal cells or splenocytes [114]. However, γHV68 Bcl-2 mutant viruses exhibited significantly impaired ex vivo reactivation from latency in peritoneal cells in both wild-type and IFN-γ $^{-}/_{-}$ mice, displaying a four- to fivefold reduction in reactivation efficiency compared to wild-type γHV68 virus. Gamma herpesvirus can also persistently replicate in cells independent of a true latent state and it has been speculated that persistent replication may occur following reactivation from latency. Consistent with the role of viral Bcl-2 in

reactivation from latency, immunocompromised IFN-γ −/− mice infected with Bcl-2-deficient γHV68 showed significantly impaired persistent replication in contrast to wild-type virus, demonstrating that γHV68 Bcl-2 is required for viral persistence. Persistent replication of γHV68 in smooth muscle cells of chronically infected IFN-γ −/− and IFN-γ receptor (IFN-γ R) −/− mice leads to severe large vessel vasculitis and death [115,116]. Because of the finding that γHV68 Bcl-2 is required for persistent replication, γHV68 Bcl-2 mutant viruses are predicted to display decreased virulence in this model of chronic disease. This was indeed the case, with IFN-γ R −/− mice chronically infected with γHV68 Bcl-2 mutant virus exhibiting approximately threefold greater survival than mice infected with virus containing functional γHV68 Bcl-2 [114]. A corresponding decrease in the incidence of arteritic lesions (74% to 28%) was also observed. Consistent with the absence of a role for γHV68 Bcl-2 in acute infection, no reduction in virulence was observed in a mouse model of acute lethal meningitis or in a recombination activating gene 1 (RAG-1)-deficient mouse model of acute disease following infection with the mutant γHV68 Bcl-2 virus [114]. Although tumors in mice infected with γHV68 have been observed on rare occasions, γHV68 does not appear to be tumorigenic in mice. Therefore, while this mouse γ-herpesvirus is a valuable tool to study pathogenesis, a model that mimics the γ-herpesvirus-associated tumors in humans is still lacking.

Several important implications arise from this study of γHV68 Bcl-2 function in vivo. First, the specific role of γHV68 Bcl-2 in persistent but not acute replication implies that persistent replication is a genetically distinct process from replication during acute infection in that different viral proteins are involved. γHV68 cyclin was similarly shown to be important for ex vivo reactivation from latency and persistent replication but not for acute replication [114,117]. Second, cell cycle progression is likely required for ex vivo reactivation from latency and γHV68 Bcl-2 may be important for preventing apoptosis induced by either viral cyclin or a host response to cell cycle activation. Such a relationship has been reported for Kaposi's sarcoma-associated herpesvirus, where expression of KSBcl-2 blocks apoptosis induced by overexpression of KSHV viral cyclin [118]. γHV68 viral cyclin is oncogenic and promotes cell cycle progression in primary lymphocytes despite causing increased apoptosis [119], suggesting that γHV68 Bcl-2 might be needed to lead to efficient immortalization in an analogous fashion to the cooperativity observed between c-*myc* and cellular Bcl-2 [17,120]. Third, persistent replication likely contributes to spread of virus within the population and it has been suggested that it may contribute to tumorigenesis by γ-herpesviruses [114,121]. Therefore, γHV68 Bcl-2 may indirectly contribute to tumorigenesis because of its importance in persistent replication even if it lacks overt oncogenic activity. Finally and most significantly, this study conclusively identifies a role of viral Bcl-2 in the virus life cycle and an associated disease state.

5.2. EBV BHRF1 and BALF1

EBV BHRF1 was the first viral Bcl-2 homolog to be identified [18]. Similar to the C-terminal hydrophobic tail of Bcl-2, the C-terminal region of BHRF1 targets the protein to intracellular membranes, including the mitochondrial outer membrane [122–124]. In keeping with other anti-apoptotic Bcl-2 proteins, BHRF1 is protective against many death stimuli, including serum deprivation and calcium ionophore treatment in B lymphocyte cell lines [19], death receptor signaling [125–127], DNA damaging agents [128], and Sindbis virus infection [90].

EBV encodes a second Bcl-2 homolog originally dubbed BALF1 [129]. Later it was recognized that translation of the BALF1 mRNA is likely initiated from the second in-frame methionine (probably the first methionine in the mRNA transcript) that, in contrast to the first methionine, is conserved in the BALF1 reading frame of several primate lymphocryptoviruses [94]. The original longer BALF protein was renamed BALF0 and the protein product resulting from translation at the internal methionine was subsequently referred to as BALF1. Overexpressed BALF0 was initially reported to confer anti-apoptotic activity [129]. However, using similar or alternate strategies, we have been unable to detect significant anti-death activity with either BALF0 or BALF1 [94]. Although BALF1 is more distantly related to cellular Bcl-2, the conservation of BALF1 in a wide range of gamma herpesviruses indicates that BALF1 has a critical function presumably related to control of cell death though other possibilities remain. The only function of BALF1 uncovered thus far is its ability to negate the anti-apoptotic activity of the other EBV viral homolog BHRF1 as well as the viral protein KSBcl-2 but not cellular Bcl-X_L. Although BALF1 and BHRF1 heterodimers can be detected in cell lysates containing detergents, distinct intracellular localization patterns were observed by immunofluorescence in both healthy and dying cells, with BALF1 exhibiting diffuse cytosolic staining in contrast to the punctate mitochondrial localization of BHRF1 [94]. Therefore, BALF1 may inhibit the anti-death activity of BHRF1 without direct physical interaction.

A role for either BHRF1 or BALF1 in EBV infections has yet to be reported. BHRF1 is expressed during the productive/lytic replication cycle [123,130]. Although latent cycle transcripts that contain the BHRF1 open reading frame have been reported [122,131,132], the encoded protein has not been convincingly detected in latently infected cells. It has been postulated that like adenovirus E1B 19K, BHRF1 may function in EBV infections to promote viral replication by preventing premature death of the host cell [4,5]. BHRF1 can functionally substitute for E1B 19K in preventing apoptosis induced by infection with an adenovirus mutant that lacks this protein [128] and apoptosis is

known to limit replication in the case of adenovirus. However, initial studies with mutant EBV viruses lacking intact BHRF1 did not display any impairment in infection and replication in B cell lymphocytes and cell lines in vitro [133,134], consistent with in vivo results obtained with γHV68 virus deficient in viral Bcl-2 [114]. The lack of a phenotype for BHRF1 ablation may be due to redundancy of anti-apoptotic function encoded by the virus. Alternatively, the finding that EBV does not efficiently enter lytic cycle replication in cultured cells has hampered efforts to delineate the role of BHRF1 in the virus life cycle. New methods have been developed by Hammerschmidt and colleagues to engineer mutations in EBV genomes and to study their effects in culture. These approaches are likely to provide new information about the functions of BHRF1 and BALF1 during infection. However, the lack of a good animal model to study EBV pathogenesis remains an obstacle to progress. An important role for BHRF1 in the EBV life cycle is predicted from the finding that BHRF1 is conserved at both the sequence and functional level in a wide range of primary EBV isolates [80]. It may be that BHRF1, like its γHV68 homolog, is involved in reactivation from latency or persistent replication, processes difficult to model in vitro. One could speculate that BALF1 allows the virus to fine-tune the regulation of host cell apoptosis so that later in the lytic cycle when death is potentially advantageous to promote the spread of progeny virus, the function of BHRF1 can be eliminated by BALF1.

EBV is closely associated with several tumors of both lymphoid and epithelial origin, including Burkitt's lymphoma, B-lymphoproliferative disease, Hodgkin's lymphoma, some T-cell lymphomas, nasopharyngeal carcinoma and stomach cancers [135,136]. The majority of the world's population is infected with EBV, which is believed to persist in latently infected B cells and become transmitted to susceptible individuals via lytic replication in the oral or genital epithelia [135]. Cytotoxic T cells are important in controlling and restricting EBV infections in healthy individuals and infection alone is insufficient for contraction of EBV-associated malignancies. The ability of BHRF1 to inhibit death induced by death receptor ligands and activated monocytes raises the possibility that BHRF1 may assist in evading the host immune response [125–127].

Immunosuppressed individuals have an increased propensity for developing EBV-associated lymphomas, however, the mechanism of tumorigenesis is not understood. Cellular Bcl-2 can cooperate with c-*myc* to induce transformation of pre-B and B lymphoid cells [17,120], and it has been shown that BHRF1 can facilitate transformation by the adenovirus protein E1A by inhibiting apoptosis [93]. Furthermore, c-*myc* translocations are observed in African Burkitt's lymphoma, which is almost always associated with EBV [137,138]. However, early studies showed that EBV with a mutated BHRF1 reading frame was still fully competent to initiate immortalization of B cells in vitro, and as stated above, BHRF1 protein expression has not been convincingly detected during latency, disfavoring a direct role for this protein in oncogenesis, at least as revealed in this model. Latent membrane protein 1 (LMP1) is expressed by EBV during latency and activates expression of the cellular *bcl-2* gene, perhaps superceding a need for the viral homolog in tumorigenesis, though there is a lack of correlation between LMP1 expression and cellular Bcl-2 expression in some cases [139–141]. It may be that BHRF1 contributes indirectly to tumorigenesis by promoting persistent viral replication and thus increasing viral load. Elevated levels of lytic cycle antigens are detected prior to the onset of several EBV-associated tumors, suggesting high viral load correlates with disease [142].

5.3. KSBcl-2

Like EBV, human herpesvirus 8, commonly called Kaposi's sarcoma herpesvirus (KSHV), is associated with the development of human cancers. The virus was first identified in the Kaposi's sarcoma (KS) skin lesions of AIDS patients [143], hence its name, and in addition to Kaposi's sarcoma, KSHV is a risk factor in the development of primary effusion lymphomas and Castleman's disease [144,145]. Like its EBV counterpart BHRF1, the Bcl-2 protein of KSHV, KSBcl-2, is expressed early in the lytic replication cycle and can inhibit apoptosis induced by several stimuli, including Bax overexpression, viral cyclin overexpression, and Sindbis virus infection [89,118,146].

KS develops from spindle cells latently infected with KSHV. Like other gamma herpesviruses, KSBcl-2 RNA is expressed upon activation of the lytic cycle in cultured cells but the issue of KSBcl-2 expression at the protein level needs further exploration. Interestingly, detection of KSBcl-2 RNA and protein has been reported in KS lesions from AIDS patients [147,148]. It was suggested that KSBcl-2 protein is widely expressed in spindle cells of late-stage nodular lesions where the number of lytically infected spindle cells was assumed to be low, implying possible expression of KSBcl-2 during latent or persistent infections [148–151]. In contrast to KS lesions, KSBcl-2 protein was not detected in stimulated/reactivated primary effusion lymphoma (PEL) cell lines despite the expression of KSBcl-2 mRNA [148]. However, the high expression levels of human Bcl-2 in these PEL cell lines suggest that the virus may not require expression of its own Bcl-2 protein, in contrast to the spindle cells in KS tumor nodules where only low levels of human Bcl-2 expression were reported [148]. Thus, KSBcl-2 expression may be similar to the latent phase expression of mouse γHV68 Bcl-2, and if confirmed, will also emphasize that expression patterns in vivo can differ from those observed in cell culture models. However, it is unknown whether KSBcl-2 expression is causally linked to development of Kaposi's sarcoma. Nor is it known if KSBcl-2 is important for maintaining cell viability during lytic cycle replication or plays a role in reactivation efficiency.

5.4. Adenovirus E1B 19K

The first indication that evading cell destruction was critical to viral infection came with the discovery of human adenovirus mutants that exhibit cytopathic effects and cellular DNA degradation following infection, termed the *cyt/deg* phenotype [152–157]. These cell death-inducing virus mutants produced ~ 10-fold fewer progeny compared to wild-type virus and were defective in E1B 19K [153,157–159], indicating a role for E1B 19K in cell death suppression and viral replication. Limited sequence homology may exist between the highly conserved BH1 domain of Bcl-2 and the human adenovirus E1B 19K protein [9]. The functional equivalence of E1B 19K to Bcl-2 in the prevention of cell death during productive infection was demonstrated by a complementation approach. Overexpression of Bcl-2 in HeLa cells or CHO cells protected against E1B 19K mutant adenovirus-induced apoptosis [9,160] and a recombinant adenovirus in which Bcl-2 substituted for the lack of E1B 19K was successful in suppressing apoptosis [161]. Like Bcl-2, E1B 19K inhibits cell death induced by diverse stimuli such as TNF-α, Fas, DNA damaging agents, ultraviolet radiation, nerve growth factor (NGF) deprivation, and the tumor suppressor protein p53 [160,162–170]. Also like cellular Bcl-2, the adenovirus E1B 19K protein can interact with a broad range of Bcl-2 family members, including Bax [52,53], Bak [51], Bik [48,49], and Nip proteins (e.g. BNIP3) [50], although it fails to interact with Bcl-2-interacting BH3-only proteins Bad [52] and Bid [42].

Although there is not a great deal of data addressing the molecular mechanism of cell death inhibition by viral Bcl-2 sequence homologs, considerable progress has been made toward understanding the function of adenovirus E1B 19K following viral infection or host death receptor signaling. Surprisingly, although Bax activation occurs both in apoptosis induced by adenovirus lacking E1B 19K and apoptosis induced by the cytotoxic cytokine TNF-α, E1B 19K only targets Bax directly in the TNF-α pathway while inhibition of virus-induced apoptosis is mediated indirectly through inhibition of Bak or unknown components of the pathway. In the TNF-α death receptor signaling pathway, E1B 19K binds directly to Bax following a conformational change at the amino terminus of Bax that is likely mediated by tBid (Fig. 1B), preventing formation of a 500-kDa Bax complex and apoptosis [43]. A requirement for direct binding of E1B 19K to Bax to inhibit apoptosis was consistent with earlier suggestions [53,171]; however, in apoptosis induced by infection with E1B 19K-deficient adenovirus, Bid is not cleaved and E1B 19K binding to Bax does not occur [44,45,107], indicating that E1B 19K possesses an alternate means of suppressing cell death.

Cuconati et al. [44] provided an explanation for the ability of E1B 19K to block apoptosis in the absence of Bax interaction by showing that E1B 19K can target Bak during adenovirus infection and prevent the downstream activation of Bax. Following infection with adenovirus lacking E1B 19K, a conformational change in Bak occurs, followed by Bak–Bax interaction, and a subsequent shift in the C-terminal conformational status of Bax. Co-immunoprecipitation data between E1B 19K and Bak and lack of Bak–Bax interaction in E1B 19K-proficient virus infection suggest that E1B 19K anti-apoptotic potency may be linked directly to its ability to bind Bak following infection. Nevertheless, the presence of E1B 19K still inhibited apoptotic DNA fragmentation in adenovirus infection of Bak-deficient cells [44], indicating that E1B 19K binding to Bak cannot be its sole means of protection during productive infection and that other mechanisms of achieving Bax activation can be recruited in the absence of Bak. Although Bid is not activated in the death pathway induced by adenovirus in the absence of E1B 19K, other BH3 proteins potentially participating in initiating apoptotic events upstream of Bax and Bak represent additional possible targets of E1B 19K.

An independent study using a human epithelial colon cancer cell line HCT116 found that Bax is required for efficient adenovirus-induced apoptosis but, in agreement with the earlier study, binding between Bak but not Bax and E1B 19K was observed [107]. Interestingly, an N-terminal truncated version of Bax was generated upon infection with E1B 19K-deficient adenovirus and the appearance of this cleavage product was inhibited by the presence of E1B 19K (i.e. in infection with wild-type adenovirus). The presence of E1B 19K also reduced the insertion of the 18-kDa truncated Bax into mitochondrial membranes. The N-terminal modification of Bax is hypothesized to liberate the C-terminal transmembrane domain from the hydrophobic cleft [84,172–174], enabling insertion of Bax and release of mitochondrial apoptogenic factors from the intermembrane space.

In contrast to human epithelial colon cancer HCT116 cells that lack Bax and exhibited reduced apoptosis following infection with E1B 19K-defective adenovirus [107], apoptosis was still observed in Bax-deficient transformed baby mouse kidney (BMK) cells expressing Bak that are semipermissive to adenovirus type 5 replication [44]. As E1B 19K is not effective at protecting adenovirus-infected mouse cells from TNF-induced death although it is fully competent to protect human cells from the same death stimulus [168], it may be that there are variations in the adenovirus-induced apoptotic pathway in mouse cells vs. human cells. Alternatively, the difference between the study using HCT116 cells and the study using BMK cells may reflect a difference between cell types as Bax and Bak have been shown to be functionally redundant in many [175] but not all cell types [176]. Despite these differences, infection of Bax/Bak doubly-deficient BMK cells with E1B 19K-deficient adenovirus significantly impaired apoptosis, coinciding with a 10-fold enhancement in viral replication [44]. This finding provides strong experimental support for the widespread belief that premature apoptosis limits viral replication. A dramatic decrease in the immunodetection

of E1A, structural polypeptide III, and other structural proteins in wild-type BMK cells infected with E1B 19K mutant adenoviruses compared to the wild-type strain indicates that progression to the late stages of the adenovirus life cycle only occurs when apoptosis is inhibited. Staining for structural proteins was observed upon infection of BMK Bax/Bak-deficient cells with the same E1B 19K mutant viruses, excluding a requirement for an alternate function of E1B 19K as an explanation for the lack of structural protein staining in cells infected with the mutant viruses. The study of the E1B 19K-deficient adenovirus in human epithelial cells lacking Bax by Lomonosova et al. [107] also revealed a critical role for E1B 19K in promoting viral replication. When viral replication was measured by single-step growth curve analysis, E1B 19K defective viruses exhibited delayed replication relative to wild-type virus in Bax-positive but not Bax-negative cells, suggesting that enhanced apoptosis in E1B 19K mutant virus infection restricts replication. Thus, the study of adenovirus E1B 19K mutant virus infection in cells defective in core apoptotic machinery has enabled the identification of the critical role of apoptosis inhibition in the virus life cycle. Additionally, it has furthered the dissection of biochemical pathways utilized by the host cell to induce apoptosis in response to infection and limit replication and viral dissemination.

5.5. Viral mitochondria-localized inhibitor of apoptosis (vMIA)

A Bcl-2 homolog has been identified in an alpha herpesvirus of turkeys, but no recognizable Bcl-2 homologs are encoded by β herpesviruses, such as human cytomegalovirus (CMV). Nevertheless, infection of human fibroblasts with CMV protects against apoptosis induced by adenoviruses lacking anti-apoptotic E1B 19K, demonstrating anti-apoptotic activity and suggesting that CMV might also express a protein with Bcl-2 like function [177]. vMIA encoded by UL37 exon 1 (UL37x1), was uncovered in a screen of a genomic CMV DNA library for clones able to rescue HeLa (human epithelial cervix carcinoma) cells from Fas-induced apoptosis [10]. This 163-amino-acid protein is encoded by the UL37 gene, along with other alternatively spliced longer variants. These longer forms, designated $gpUL37_M$ and gpUL37, share the initial 162 amino acids with pUL37x1 and also possess limited anti-apoptotic activity, albeit much less robust than pUL37x1. Immunofluorescence and immunoelectron microscopy studies revealed a predominantly mitochondrial outer membrane localization for vMIA, prompting the redesignation of pUL37x1 as vMIA [10]. An antisense oligonucleotide approach has demonstrated that the UL37 gene is required for human CMV DNA replication [178], however, whether the anti-apoptotic activity of vMIA is responsible for this requirement is currently under investigation.

There is no amino acid sequence similarity between vMIA and Bcl-2 family members, and vMIA interactions with Bcl-2 family members have yet to be published. Two domains within vMIA are important for its ability to block cell death, one near the N-terminus (amino acids 5–34) that is required for mitochondrial targeting and the second spanning amino acids 118–147 that is of unknown function but may be important for the observed interaction between vMIA and the ANT [179,180]. The interaction of vMIA with the ANT may be protective by inhibiting mitochondrial permeability transition and cytochrome *c* release or by regulating ADP/ATP exchange as has been proposed for Bcl-2 [63,67,181].

vMIA was also found to alter mitochondrial morphology. Expression of a dominant-negative version of the mitochondrial fission protein Drp-1 inhibits mitochondrial fragmentation during apoptosis, delays cytochrome *c* release, and delays cell death [182,183], prompting investigation of changes in mitochondrial morphology during apoptosis. A caspase-8 cleavage product of Bcl-2-associated protein-31 (BAP31) at the ER promotes ER-to-mitochondria calcium signaling that recruits Drp1 to mitochondria [184]. The recruitment of Drp1 leads to mitochondrial fission and sensitization to tBid-mediated cytochrome *c* release. Surprisingly, examination of mitiochondrial morphology following CMV-infection of human fibroblasts revealed a fragmentation of mitochondrial networks that is apparently mediated by vMIA [185]. Paradoxically, vMIA-induced mitochondrial fragmentation is strikingly similar to that observed during apoptosis. In contrast to what was expected from the correlation between mitochondrial fission and tBid-mediated death, CMV-infected cells with fragmented mitochondria were protected against Fas-induced death. A 68-amino-acid peptide encompassing the two identified functional domains of vMIA was fully competent to protect cells and also to induce the fragmented mitochondrial phenotype, suggesting a link between mitochondrial fission and anti-apoptotic activity [185]. Cells with fragmented mitochondria expressing the highest levels of vMIA displayed fainter Mitotracker Red® staining compared to cells with lower vMIA content and more reticular mitochondrial morphology [185]. As Mitotracker Red® uptake is dependent on mitochondrial membrane potential, this suggests that mitochondria that underwent fission have a reduced membrane potential. It is therefore possible that a lowered mitochondrial membrane potential contributes to protection by vMIA since there is an inverse relationship between membrane potential and the production of reactive oxygen species [186]. The functional role of mitochondrial fragmentation in apoptosis is still unclear, however, and it is possible that premature or altered fission may impede the apoptotic process.

6. Conclusions

It has been widely hypothesized that viral Bcl-2 homologs serve a vital role in the virus life cycle by inhibiting the

premature apoptotic death of host cells during acute replication, allowing completion of the replication cycle and favoring the spread of progeny virus. Although it appears that the adenovirus functional Bcl-2 homolog E1B 19K indeed functions in this manner, the limited evidence on the function of γ-herpesvirus Bcl-2 proteins does not support a similar role. This may be due to the redundancy of apoptosis inhibitors encoded by many of the viruses that contain Bcl-2 homologs or, alternatively, viral Bcl-2 proteins may participate in the virus life cycle in more complex ways. A study of murine γHV68 Bcl-2 demonstrates a role for this protein in reactivation from latency, persistent replication, and chronic disease, indicating that viral Bcl-2 proteins may indeed play a significant role in virulence. It may turn out that different viruses need Bcl-2 homologs to block apoptosis in different aspects of their life cycle and that there is no one unifying hypothesis that fits all viral Bcl-2 proteins. Undoubtedly, concerning the role of viral Bcl-2 homologs, we still have much to learn.

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